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AUTHORITY

BDRL, D/A ltr, 13 Sep 1971

TRANSLATION NO. 37 CB

DATE: July 1968

(1)

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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

Revue d'Immunologie 11: 206-221. 1947

#37 G.1

Role of Phagocytic Defense in the Mechanism of Action of Streptomycin in Vivo.

by

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The object of the present memoir is the precision of the role played by the natural defenses of the organism, in particular phagocytosis, in the final act of the process which assures annihilation of pathogenic micro-organisms in animals submitted to treatment by streptomycin. A general law is involved, regulating the therapeutic action of sulfamides as well as penicillin. In effect, as concerns sulfamides, it has been shown in experiments carried out with pneumococcus, streptococcus, B. Friedlander and other bacteria, that if it is proved that "efficacious medications exercising in vivo an incontestable bacteriostatic action, we must concede that another factor finishes the work of bacteriostatis. Now this factor can only be phagocytosis. It is this which has the last word in the conflict which the organism, under the chemotherapeutic shock, engages with the invader. It is this which assures, thanks to the englobement of germs and their intracellular digestion, the definite annihilation of the pathogenic agent". "The intervention of the phagocytic apparatus in the epilogue of the drama is a proof, nonetheless demonstrable, pleading for intervention of organic defenses in the

chemotherapeutic warfare of microbial diseases". (C. Levaditi, A. Vaisman and D. Krassnoff (1); C. Levaditi (2).)

Besides, experiments with staphylococcus, hemolytic streptococcus, pneumococcus, submitted in vivo to the curative effects of penicillin, have led to conclusions analogous to the preceding. "Finally, it is especially thanks to ultimate intervention of the defensive system of the organism, and in particular to phagocytosis, that the sterilizing activity of penicillin is exercised". (C. Levaditi and A. Vaisman (3).)

Is it also the mechanism of action of streptomycin? Our experimences have been with Staphylococcus aureus. Escherichia coli, Klebsiella pneumoniae (Friedlander's Bacillus) and Bacillus anthracis, following the technique here given:

Technique. — Series of mice are inoculated, by intraperitoneal route, with lethal doses of cultures aged 24 hours on bouillon (0 cmc.3). Some serve as controls, the others are treated (subcutaneous route) with 1,000, 2,000 or 5,000 micrograms of antibiotic (according to the species of microbe being considered), in 2 or 3 injections more of less spaced. At regular intervals the mice belonging to each one of these series are sacrificed (except for those which succumb). Examinations bear on the peritoneal exudate and on the blood. For coeliac liquid, cultures and smears are made; the latter are stained with phenolised methylene blue or by process permitting the evidence of capsules. Blood cultures furnish information

on the presence or absence of septicemia.

From the quality, the content of peritoneal exudate is evaluated in free germs by direct count (rate of microscopic field) and the quantity and quality of phagocytes, intensity of phagocytosis is determined, even the fate of englobed germs. The collection of this data serves to establish graphs, or the number of bacteria free per field is indicated by figures varying from zero (absence of bacteria) to infinity (more than 100), and where the degree of phagocytosis, as well as the type of phagocytes(polynuclear, mononuclear or gross macrophages) figure schematically. Even the capsulation and involutive modifications of these microbes are seen.

Here are the results obtained:

I. STAPHYLOCOCCUS AUREUS.

1. Control mice (graph I). — Inoculation by intraperitoneal route of 0 cmc 3 Culture on boudillon, aged 24 hours. The deaths take place between the 14th and 36th hour.

a) Bacterial content of peritoneal exudate

Time (in hours)	Number of Microbes per field
0 - 1	1
2	3,4
4	19
6	00

Thus, progressive augmentation in the number of germs, which reach infinity in six hours.

b) Leucocytosis and phagocytosis. — Leucocytosis is constituted exclusively by polynuclears. These are in a state of lysis in dead animals. Phagocytosis passes from ++ at the fourth hour to ++++ at the sixth hour. But at this last period of infection, the englobed germs prepare a leucolysin due to an intense leucolytic power.

c) Cultures of peritoneal liquid are revealed positive during the whole experiment; blood cultures become so only at the eighth hour.

These experiments show that in control mice survival is nil, de death staphylococci in the peritoneal fluid and septicemia beginning at the sixth hour. Phagocytosis, inoperative because of the lysis of leucocytes, itself an effect of staphylococcic leucolysins, offers no resistance to multiplication of microorganisms, whence the early death of the animals.

2. Treated mice (Graph 2). — Treatment with 3 x 1000 micrograms (immediately, 4 hours and 8 hours after the infection). Definite survival of the animals.

a). Bacterial content of peritoneal exudate:

Time (in hours)	Number of germs per field
0	> 26
30	1
1	0.2
2	0.2
6, up to 7th day	< 1
	0

Thus, progressive disappearance of germs, becomes total at the sixth hour, continuing thus to the seventh day.

b). Leucocytosis and phagocytosis. Leucocytosis is rapid the rare lymphocytes verified at the beginning of the experiment being replaced by polynuclears first, by an association of white globules with lobed nuclei and macrophages at the beginning of the fourth hour. Phagocytosis is intense between the second and the eighth hour, to be attenuated between the tenth and forty-eigth hour and disappear completely after the third day. Englobement is accomplished, first by polynuclears, lastly by large mononuclears. The latter morphological and tinctorial modifications, representing a whole series of involutive phenomena, up to the final stage of total intracellular lysis. Besides, the macrophages englobe polynuclears containing phagocytic germs. (Phenomenon already observed with penicillin).

c). Cultures. — Cultures of peritoneal exudate remain positive pp to the sixth day, while those of blood have been negative during the whole course of the experiment (except the fourth day). This shows that streptomycin hinders the appearance of staphylococcic septicemia, but does not sterilize the peritoneal cavity until the very end of the experiment. The reason for it is that the leucocytes having englobed the microorganisms in a state of bacteriostasis, but still living, protect them from the germicidal effect of the antibiotic, while anticipating that they

assure total lysis even in the midst of their cytoplasm.

It is to be concluded that phagocytosis, principal factor of defense of which the organism is capable, plays a role of prime importance in the final act of streptomycin cure of infection produced in mice by Staphylococcus aureus.

2. Escherichia coli (Bact. coli)

1. Control mice (graph 3) —Intraperitoneal inoculation of 0 cmc. 3 of a culture on bouillon, aged 24 hours. Deaths take place between the second and fourth hours. Thus, evolution is more rapid than that of staphylococcic infection.

a). Bacterial content of peritoneal exudate:

Time in hours	Number of microbes per field
0 to 30'	> 50
1	> 50
2	infinite (elongated forms)
4 to 14	infinite

Thus, rapid multiplication of microorganisms in the peritoneal cavity attains infinity by the second hour.

b). Leucocytosis and phagocytosis.— From the second hour, massive polynuclear leucocytosis. It is the same with phagocytosis which is intense, but which leads to lysis of which globules, whence its inactivity. (Note an attachment of bacteria to the surface of lymphocytes at the beginning of the experiment).

c). Cultures. Cultures of blood and coelic exudate are positive during the whole experiment, evidence of a state of early septicemia.

2. Mice treated (Graph. 4). — Treatment with 3 x 5,000 micrograms by subcutaneous route (immediately, six and 14 hours after infection)

a). Bacterial content of peritoneal exudate:

Time in hours	Number of bacteria per field
0	2
30' to 1	50
2 to 4	25
6	5
8	3,4
10	2,1
20	< 1
30 to 5 days	0

Thus, after a transient multiplication of the germs toward the end of the first hour, progressive disappearance of the germs is effected between the sixth and the twentieth hour, which, after this laps of time, leads to total effacement, lasting the duration of the experiment (five days). In the course of this process, certain B. coli show signs of degeneration, undergoing a transformation in polymorphic elements, having the appearance of stromas.

b). Leucocytosis and phagocytosis:

Time in hours	Phagocytosis
30' to 1	Trace
2	/ / / (poly)
4 to 6	/ / (poly)
8 to 10	/ / / / (poly)
20	Trace —
30	/ / / (poly and mono)
48	Trace (poly)
3 to 5 days	0 (mono)

Leucocytosis and phagocytosis are intense from the second hour. This is accomplished first by polynuclears and towards the last, by large mononuclears of the macrophage type. Now, towards the tenth hour, the englobed microorganisms offer signs of intra-cytoplasmic degeneration phenomena which preceded their integal lysis. Certain of the macrophages englobe polynuclears having phagocytized B. coli.

c). Cultures. Blood cultures are sterile during the whole course of the experiment (except at the fourth hour), those of the peritoneal cavity become radically negative only at the 8th hour, a phenomenon similar to that shown by the experiment preceding (Staphylococcus aureus).

It can be concluded, then, as concerns Escherichia coli, that phagocytosis intervenes actively in the process of healing of an infection provoked by this organism in mice.

3. Klebsiella pneumoniae (Friedlander's Bacillus)

The case of Friedlander's bacillus, like that of Bacillus anthracis, offers a particular point of interest for us. In effect, this bacillus is eminently capsulogenic. We have shown, by the mechanism of action of sulfamides and penicillin, that the capsulation of streprococcus, of pneumococcus and Klebsiella is an important factor in the efficacy of the phagocytic defense. It is in hindering capsulation, in other words the formation of this apparatus of anti-phagocytic protection which is the capsule, that medication, from the fact of their sulfamide or antibiotic activity, render certain microorganisms susceptible of being englobed and assimilated by the phagocytes.

Now, this anti-capsulogenic factor intervenes effectively in the process of healing with streptomycin of infection engendered by Klebsiella pneumoniae. It is this which results from the experiments summarized below:

1. Control mice (Graph 5). Intraperitoneal injection of 0 cmc 3 of culture on bouillon, aged 24 hours. Death in six hours.

Time in hours	Number of bacteria per field	
0 to 1	23 to 27	Partial Capsulation
2	> 50	
4	> 100	
6	Infinity	Total capsulation

Thus, an intensive multiplication of microorganisms beginning towards the second hour, to give place finally (sixth hour) to a true intra-peritoneal culture.

b). Leucocytosis and phagocytosis. — Since polynuclear leucocytosis is more marked at the beginning of the second hour, phagocytosis is less marked.

c) Capsulation. — Non-capsulated during the first two hours, the B. of Friedlander, after having passed through a short phase of polymorphism, is surrounded by capsules, encapsulation becoming integral at the sixth hour (argentic impregnation).

d) Cultures. — They are positive during the whole duration of the experiment. (blood and peritoneal fluid).

Thus, septicemia and peritonitis in encapsulated Klebsiella, occasioning a rapid death.

2. Treated mice (Graph 6). — Treatment by 2 x 2,000 micrograms (immediately and after eight hours). Definite survival of the animals.

a) Bacterial content of peritoneal fluid:

Time in hours	Number of bacteria per field	Capsulation	
0	21	0	
30' to 1 h.	17	0	
2	26	+	
4	8	+	
6	6,5	0	
8	2	0	degenerated
16 to 2 days	0	0	

Decrease in number of bacteria after the fourth hour is accented progressively thereafter; it ends in complete disappearance of these bacteria at the sixth hour and continues thus to the end of the experiment (second day).

b) Leucocytosis and phagocytosis.— Polynuclear leucocytosis is marked, to become, after the sixth hour, a macrophagic leucocytosis. As for phagocytosis, it is scarcely noted and does not appear, except in an ephemeral fashion, except at the second and the twenty-fourth hour.

c) Capsulation — As shown in the table above and in Graph 6, capsulation appears only as a tentative state, between the second and fourth hour, when the bacteria have passed through a phase of polymorphism. Then, after the sixth hour, and before they have disappeared from the exudate, the Klebsiella show signs of manifest involutive degeneration (argentic impregnation).

d) Cultures. — Cultures of blood are consistently negative during the whole course of the experiment; cultures of peritoneal exudate become so after the sixth hour.

Here, then, are the conclusions which resulted from the gathering of evidence:

1. Capsulation of Klebsiella pneumonia is a condition sine qua non of multiplication of bacteria in the peritoneal exudate, and in consequence, of mortal septicemia of treated mice.

2. Phagocytosis does not appear except for a trace and as an ephemeral incidence, in treated mice.

3. Decrease in number of free bacteria (preceded by morphological and staining modifications), so that their complete disappearance seems due to a direct bactericidal effect, and lysis exercised by a streptomycin. This effect is accompanied by annihilation of potential capsulogenicity of the bacteria.

From all this it results that, contrary to what took place with Staphylococcus aureus and Escherichia coli, phagocytosis disappears before the microbicidal activity and direct lysis exercised by streptomycin in Klebsiella pneumoniae. The importance of its role, as a factor of defense, varies then according to the microbial species under consideration.

It seems interesting to us to compare, from this point of view, the mechanism of action of sulfamides and streptomycin. The latter antibiotic has a strong bactericidal and lytic effect with regard to Klebsiella pneumoniae, while sulfamides were revealed to be exclusively bacteriostatic. This explains the important role of phagocytosis in sulfamide healing of infection caused by Friedlander's bacillus (S. Levaditi, A. Vaisman and D. Krassnoff - Bull. Acad. Med. 121: 730, 1939)

The same role disappears with streptomycin, the curative effect of which consists in a distinct preeminence of bactericide and extracellular lysis.

4. Bacillus anthracis

Here we are concerned, as in the preceding, with a capsulogenic bacteria capable of defending itself against phagocytosis thanks to its aptitude for encapsulation.

1. Control mice (Graph 60. Intraperitoneal inoculation of 0 cmc 3 of culture on bouillon, aged 24 hours. Death in 14 - 24 hours.

a). Bacterial content of peritoneal exudate:

Time in hours	Number of bacteria per field	
0	5)	
1	4)	Non-capsulated
4	14)	
10	Infinity)	
14	Infinity)	Capsulated
24	Infinity)	

Thus, the number of free bacteria in the peritoneal exudate augments progressively beginning with the fourth hour, to attain infinity and be maintained to the end of the experiment.

b) Leucocytosis and phagocytosis.— The initial lymphocytosis is rapidly replaced by polynucleosis. The polynuclears, in dead mice, are lysed. As for phagocytosis, it is fully developed toward the second hour.

c). Capsulation. — Begins toward the tenth hour and is maintained during the rest of the experiment.

d). Cultures. — Positive in blood and peritoneal exudate.

Thus, fatal septicemia determined by B. anthracis encapsulated.

2. Treated mice (Graph 8). — Treatment with 3 x 1,000 micrograms (immediately, 24 and 48 hours after infection). Definite survival.

a) Bacterial content of peritoneal exudate:

Time in hours	Number of bacteria per field	Capsulation
0	5	0
30' to 1	0,4	0 part. degenerated
2	3	0 " "
4 to 48	0	" "

Thus, progressive diminution of the number of bacteria in the peritoneal exudate and complete disappearance from the fourth hour.

b) Leucocytosis and phagocytosis. — Leucocytosis, at first lymphocytic, becomes polynuclear from the second hour and is associated with an intense mononucleosis beginning with the eighth hour. This mononucleosis is predominant toward the end of the experiment.
As for phagocytosis, it is practically nothing.

c) Capsulation. — Capsulation as such does not exist, but on the other hand, the bacteria show signs of partial or even toal degeneration beginning with the first hour.

d) Cultures. — Negative in blood and almost totally negative in the peritoneal liquid (absence of septicemia).

From all this it may be concluded that, parallel to Klebsiella pneumoniae, Bacillus anthracis, a microorganism eminently capsulogenic, effectively defends itself against phagocytosis, thanks to the protective capsules with which it is surrounded, succeeds in multiplying intensely in normal mice, in which it results in septicemia and death. In mice treated with streptomycin, on the contrary, the same microorganism undergoes an extracellular change, being manifested by loss of potential capsulogenicity and by involutive and staining alterations attributable to the direct microbicidal effects of the antibiotic. Phagocytosis seems to play no effective role in the defense of the organism against this pathogenic agent.

Conclusions

The experimental study of the mode of action of streptomycin in vivo throws light on the role of primary importance played by phagocytosis in the annihilation of certain pathogenic microorganisms. Phagocytic englobement and intracellular digestion of these microbes intervene in the last act of the fight which is waged between the organism submitted to streptomycin treatment and the microbe. This is exact in the case of Staphylococcus aureus and Escherichia coli. On the other hand, in infections caused by Klebsiella pneumoniae and Bacillus anthracis, intervention of phagocytic defense is either effaced or apparently non-existent. Here, the bactericidal and anticapsulogenic effects of streptomycin are part of the first plan, the destruction of bacteria being more the result of such effects than the intervention of phagocytes.

The degree of this intervention varies then according to the type of microbe under consideration. It appears at least probable that in the course of treatment with streptomycin the phagocytic defense is more effective in respect to non-capsulogenic microbes than face to face with pathogenic agents susceptible of encapsulation.

(Institut Alfred Fournier)